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PRINCIPAL INVESTIGATOR: Samir M. Hanash, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Michigan
Ann Arbor, Michigan 48109-1274

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Ann Arbor, Michigan 48109

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13. ABSTRACT (Maximum 200 words)

The dynamic turnover of microtubules within the cell is essential for a number of cellular processes including progression through the cell cycle and intracellular transport. A number of chemotherapeutic drugs target microtubules and disrupt their turnover. In particular, Taxol has an antiproliferative effect due to its stabilization of microtubules, disrupting microtubule transition during the cell cycle. A gene the Private Investigator has cloned designated Op18, has recently been demonstrated to play a regulatory role in microtubule transition through binding of the protein it encodes to tubulin dimers. Op18 protein destabilizes microtubules and therefore has a directly opposite effect to Taxol on microtubules. We have demonstrated high level expression of OP18 in a variety of tumors, including some primary breast cancers. We propose to determine the effect of manipulating Op18 expression in breast adenocarcinoma cell lines on microtubule stability and response to Taxol. We also propose to determine the relationship between Op18 level in primary tumors of the breast and biological characteristics of the tumors. The proposed investigations may provide novel strategies for inhibiting proliferation in breast cancer.

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FOREWORD

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Table of Contents

1. Foreword

The experiments proposed for this project have been largely accomplished. The resulting findings are the subjects of two manuscripts currently under preparation. One objective of this project was to test the hypothesis that Op18 levels as well as its phosphorylation status affect the ability of cells, particularly breast cancer cells, to progress through the cell cycle and to proliferate. As presented below, this objective has been accomplished. To test this hypothesis, we transfected different constructs containing: Op18 in the sense or antisense orientation; vector alone; or mutant Op18 into NIH 3T3 and MCF 10 breast cancer cells and examined the effect of manipulated expression of Op18 on transfected cells. Another objective of the project was to test the hypothesis that Op18 exerts a regulatory role in microtubule assembly that is dependent on both Op18 level and phosphorylation status. The testing of this hypothesis was accomplished by examining the effect of wild-type and mutant Op18 on microtubule assembly and by determining the sensitivity of cells transfected with different Op18 constructs to the microtubule stabilizing drug taxol. The project has resulted in the production of cell lines with manipulated expression of Op18. These cell lines remain as an important resource at the completion of the funded period. We intend to continue to experiment with these cell lines in the months and years to come.

2. List of appendices

Appendix 1: MTT assay results for transfected cell lines

Appendix 2: Taxol effects of wild-type and mutant Op18 transfectants

Appendix 3: Phosphorylation status of wild-type and mutant transfectants

3. Statement of the problem studied

The microtubule system has emerged as an important target of chemotherapeutic intervention in breast cancer (1-4). This proposal is intended to explore novel strategies that target microtubules that are relevant to breast cancer based on our cloning of a gene, which we designated Op18 and which was found to function as a regulator of microtubule dynamics (5-10). The dynamic turnover of microtubules within the cell is essential for a number of cellular processes including progression through the different phases of the cell cycle, intracellular transport, and maintenance of cell shape. Disruption of microtubule turnover results in inability of cells to progress through the cell cycle. An understanding of the regulation of microtubule transitions is highly relevant to the targeting of microtubules for chemotherapeutic intervention. Tubulin is an integral component of microtubules. It occurs primarily as soluble heterodimers consisting primarily of α- and ß-tubulin isoforms or as assembled tubulin polymers that form microtubules (5). A number of enzymes can induce rapid changes in tubulin structure, particularly at the highly variable carboxy terminal region of the molecule (6). Op18 is expressed at high levels in a number of tumor types including breast cancer (7-10). Studies have shown that this gene plays a critical role, through phosphorylation of its protein product, in progression through the cell cycle (11-15). Mutation of this gene at *Ser* residues, which are normally phosphorylated during cell cycle progression, results in mitotic arrest in the G2/M phase (16). The Op18 gene has been demonstrated to play a regulatory role in microtubule transition through binding of the protein it encodes to tubulin dimers (17,18). Op18 protein was found to destabilize microtubules, an effect which is diametrically opposite to that of taxol (19,20).

The objectives of our project have been to: 1) develop an understanding of the role Op18 in regulating cell cycle transition in breast cancer cells; 2) examine the effect of disrupting Op18, through mutation, on breast cancer cell growth; and 3) examine the effect of manipulating Op18 expression on response of breast cancer cells to taxol.

4. Summary of the most important results

4.1 Discovery of an Op18 mutation in a tumor

Expression of Op18 was previously found to be upregulated in a variety of neoplasms. However, no mutations were previously described. In an analysis of a series of tumors by two-dimensional (2-D) electrophoresis, one tumor displayed a novel protein which had a similar molecular mass as Op18 but with a more acidic isoelectric point, suggesting that it may be a variant of Op18 or an unrelated protein. Using an anti-Op18 polyclonal antibody, we examined whether this protein was an Op18 variant by 2-D electrophoresis, followed by Western blot analysis. Interestingly, this novel protein was recognized by the antibody, indicating a potential mutation. To identify the mutation(s) of Op18, polyA+ RNA was isolated from the tumor and subjected to RT-PCR to amplify the Op18 cDNA. The PCR products were purified and used directly for DNA sequencing analysis to avoid random mutations that might be introduced by Taq polymerase. A substitution of a G for C was detected at nucleotide 155 in the Op18 cDNA, which resulted in a glutamine to glutamic acid substitution at residue 18 and the concomitant shift in the pI. The discovery of a mutation in Op18 in a tumor provided an important means for evaluating the role of Op18 in cancer in general and in breast tumors in particular.

4.2 The effect of wild-type and mutant Op18 on cell growth and response to taxol

The identification of Op18 as a regulator of microtubules is of substantial significance in view of the large body of evidence that points to the importance of microtubules and their regulated growth or shrinkage (microtubule dynamics) in a number of essential cellular processes that include progression through the cell cycle, maintenance of cell shape, and intracellular transport. The dynamic turnover of microtubules within the cell contributes to their formation of different arrays including the radial arrangement in proliferating interphase cells, the mitotic spindle in dividing cells, and the staggered linear array in axons. The regulation of microtubule transitions during the cell cycle are likely regulated by kinases that also phosphorylate Op18 (19,20). Addition of Cdc2 kinase to cell extracts is sufficient to alter microtubule lengths from those typical of interphase to mitotic levels. Microtubules are essential for a number of cellular processes, Op18 therefore through its regulation of microtubules, appears to play an important role in cell functions that are dependent on microtubules. One objective of this project was to test the hypothesis that Op18 levels as well as its phosphorylation status affect the ability of cells, particularly breast cancer cells, to progress through the cell cycle and to proliferate. To test this hypothesis, we transfected different constructs containing: Op18 in the sense or antisense orientation; vector alone; or mutant Op18, and examined the effect of manipulated expression of Op18 on transfected cells. Another objective of the project was to test the hypothesis that Op18 exerts a regulatory role in microtubule assembly that is dependent on both Op18 level and phosphorylation status. The testing of this hypothesis was accomplished by examining the effect of wild-type and mutant Op18 on microtubule assembly and by determining the sensitivity of cells transfected with different Op18 constructs to the microtubule stabilizing drug taxol.

The mutant OP18 cDNA (as well as the sense wild-type, anti-sense and vector only constructs) were subcloned into the pTP2000 bicistronic expression vector, retrovirus was made and used to infect NIH/3T3 cells and allowed to integrate into the cell genome.

Expression of the various forms of OP18 in this vector system is coupled to neomycin resistance. Determination of proper expression of OP18 was confirmed by Western blot analysis of M-OP18, S-OP18, AS-OP18 and V-OP18 cell lysates. Cell growth rates were determined for NIH/3T3 cells expressing each of the constructs. We found that expression of the mutant form of OP18 had a marked retardation of cell doubling times, from about 23 hours for the S-OP18, AS-OP18, and vector only controls, to about 34 hours with the M-OP18. Additionally, the M-OP18 cells exhibited lower cell density than S-OP18 when grown in reduced serum (Appendix 1). Flow cytometric analysis of NIH/3T3 cell infectants (M-OP18 and S-OP18) revealed that the percentage of mutant-containing cells in G2/M was approximately double that which was seen with S-OP18, suggesting the prolonged doubling times observed with M-OP18 were due to a G2/M blockage. Thus, we explored whether addition of taxol (which in sufficient concentration will itself cause a G2/M blockage of the cell cycle) would further perturb cell cycle progression of M-OP18 as compared with S-OP18. We found that intermediate levels of taxol (i.e., those which do not cause a complete block in G2/M) caused the appearance of a novel sub-G1 peak, which may either be caused by apoptosis or segregation of chromosomes to multipolar (more than two) spindles during mitosis (Appendix 2). These results suggest that M-OP18 may enhance stability of microtubules through the cell cycle. The appearance of the novel sub-G1 peak is not seen in metaphase spreads of the taxol treated cells, However, metaphase spreads only look at cells in the 4N stage. If the sub-G1 peak resulted from a recent mitotic event, and the cell had not yet cycled (if it were able) all the way back through the cell cycle, the cell containing diminished amounts of DNA would not be evident by this technique.

4.3 Oncogenicity of mutant Op18 and effect on phosphorylation

We ascertained the ability of both M-OP18 and S-OP18 to grow in an anchorage-independent fashion (i.e., in soft agar), a hallmark of the tumorigenic state. We found that only the M-OP18 cells formed colonies in soft agar. Furthermore, when cells expressing M-OP18 were introduced sub-Q into SCID mice, tumors greater than 5 mm in diameter appeared within 21 days. In contrast, no tumors appeared in mice injected with S-OP18. Cell lysates were prepared from each tumor and protein expression was analyzed by 2-D PAGE. The tumors did express the mutated form of OP18. Pathologically, these tumors appeared as high-grade neoplasms with areas of high-grade adenocarcinoma (as evidenced by glandular formation) differentiation. We are still awaiting cytogenetic analysis of cells derived from these tumors.

As the mutated form of OP18 involved an amino acid substitution immediately adjacent to two key phosphorylation sites, we postulated that the mutation might have resulted in decreased levels of phosphorylation of the mutant protein as compared with the wild-type form. Overall, we found that the levels of both the mono- and bi-phosphorylated forms of M-OP18 were substantially reduced as compared with these phosphorylated forms of wild-type OP18, while the levels of all other cellular phosphoproteins detected remained invariant. To further explore which of the phosphorylation sites on the M-OP18 was adversely affected by the mutated phenotype, Western blot analysis of both the M-OP18 and S-OP18 was performed, using site-specific rabbit antibodies to phosphoserine 16, phosphoserine 25, and phosphoserine 38 (an antibody to phosphoserine 63 was unavailable) (Appendix 3). We have found that phosphorylation of serine 38 was largely unaffected by the mutant phenotype as compared with the wild-type phenotype. In marked contrast, however, phosphorylation of serine 25 in M-OP18 was greatly reduced when compared with that observed on wild type. Additionally, there was no phosphorylation of serine 16 detectable in M-OP18, although we were able to detect it in S-OP18.

4.4 Op18/tubulin interactions

As the dephosphorylated form of OP18 has been shown to bind to microtubules and stabilize tubulin dimers, we sought to ascertain whether expression of the mutated form of OP18 would lead to increased stabilization of microtubules with associated changes in microtubular ultrastructure in cells as compared to that visualized with S-OP18. Both M-OP18 and S-OP18 cells were stained for either α-tubulin, ß-tubulin or γ-tubulin (found in centrosomes) immunofluorescence, in the presence or absence of immunofluorescent staining of OP18. Overall, we observed unusual rings and swirls, and on occasion intense punctate spots, of both α- and ß-tubulin immunoreactivity near the cell periphery in the NIH/3T3 cells expressing M-OP18. Similar structures have never been visualized in NIH/3T3 cells expressing S-OP18. Further, we did not find any differences in the numbers of centrosomes or in their subcellular location between M-OP18 and S-OP18 infectants, as visualized by γ-tubulin immunofluorescence. The rings, swirls and intense spots of α- and ß-tubulin immunoreactivity co-localized with immunofluorescent staining patterns observed with anti-OP18 antibodies, suggesting that OP18 was associated with these structures. The immunoreactive structures appear to be composed of polymerized tubulin molecules (e.g., microtubules), as brief treatment of M-OP18 cells with the microtubule depolymerizing agent nocodazole caused their complete disappearance. Therefore, we explored whether the tumor of the gastric cardia from which the mutated form of OP18 was cloned would also demonstrate unusual tubulin immunoreactivity. Frozen sections of this tumor, as well as from a different gastric cardia adenocarcinoma, an esophageal adenocarcinoma, and normal gastric mucosa, were obtained and stained for both α- and ß-tubulin. We observed numerous punctate spots of intense immunoreactivity in only the tumor with the OP18 mutation, suggesting that the structures visualized in the infected NIH/3T3 cells actually resulted from the OP18 mutation. However, we did not find a measurable increase in the amount of polymerized tubulin (either α, ß or γ) in the cells expressing the mutant phenotype.

We have tried to determine what else OP18 (either the wild type or the mutant type) may be interacting with, in light of perhaps an altered interaction with the mutant OP18 leading to induction of tumorigenesis in the patient. To this end, we have performed co-immunoprecipitations (immunoprecipitate OP18, resolve immunoprecipitate by SDS-PAGE, silver stain the gel, and evaluate what else might have been precipitated with OP18). We have found a very large protein (it migrates slower than myosin, which is 220 kDa), which is enriched in immunoprecipitates from cells overexpressing the wild-type protein. It is plausible that the mutant OP18 is unable to interact with it, thus leading to a significantly lower level in the M-OP18 immunoprecipitate. At present, we are attempting to determine its identification.

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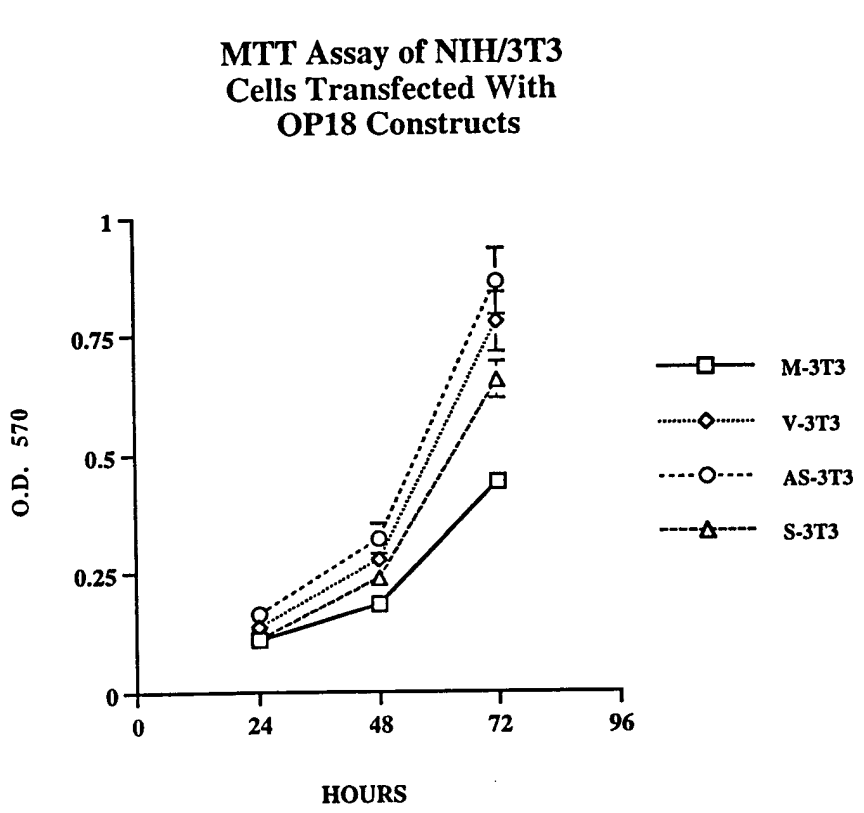
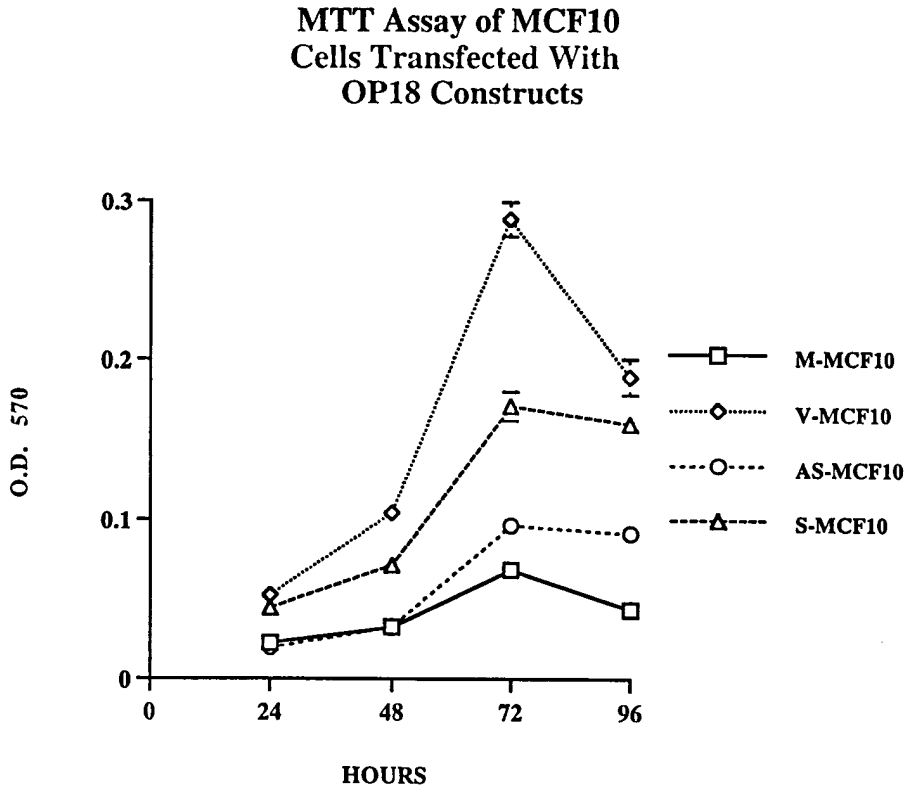
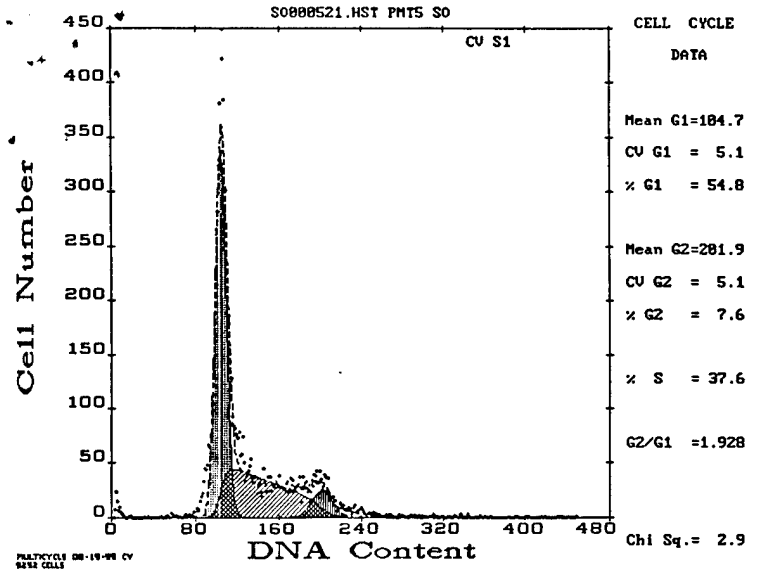
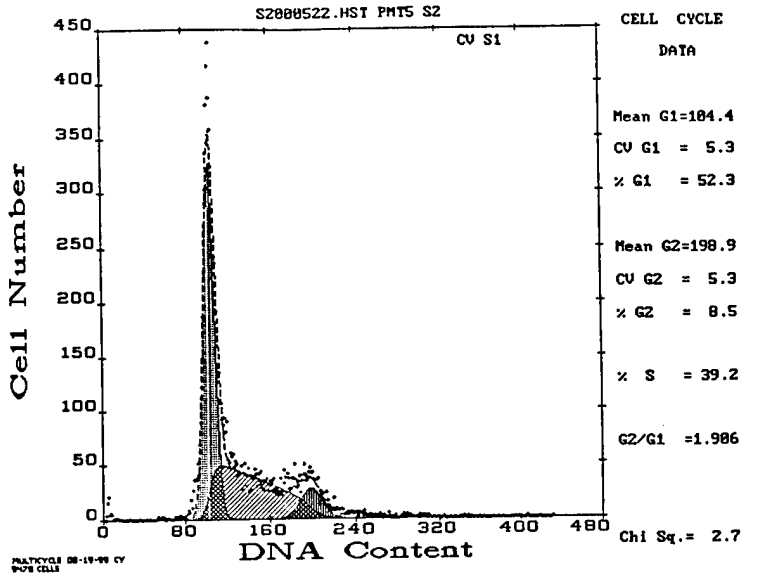
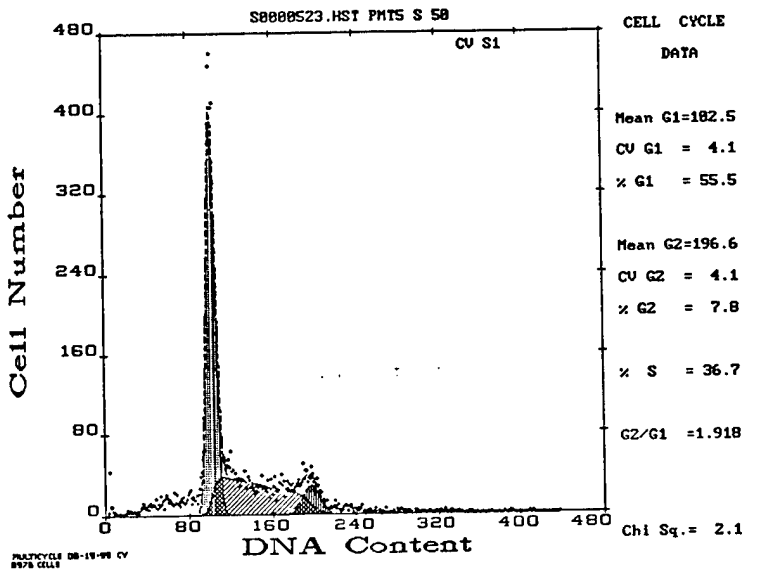


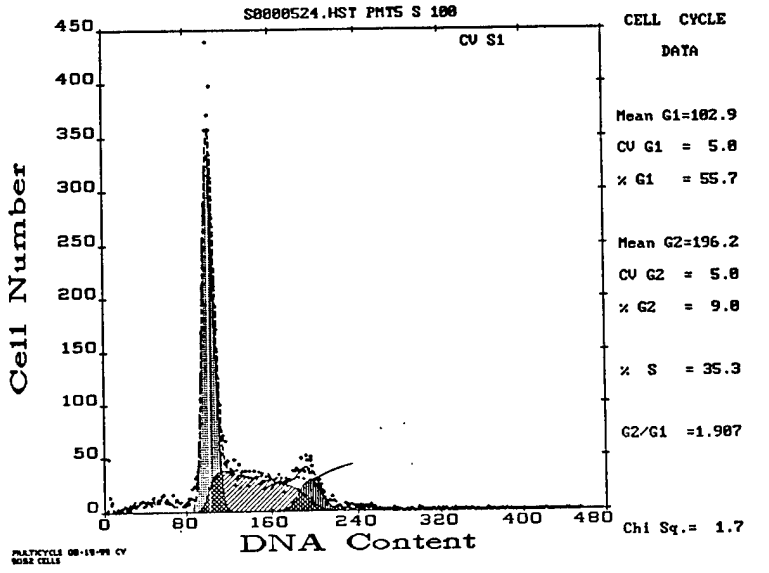


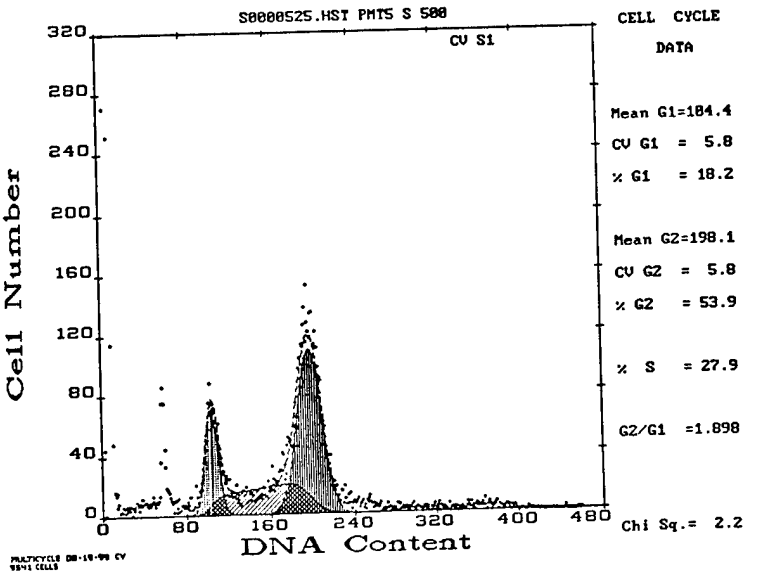
Appendix 1. MTT Assay Results

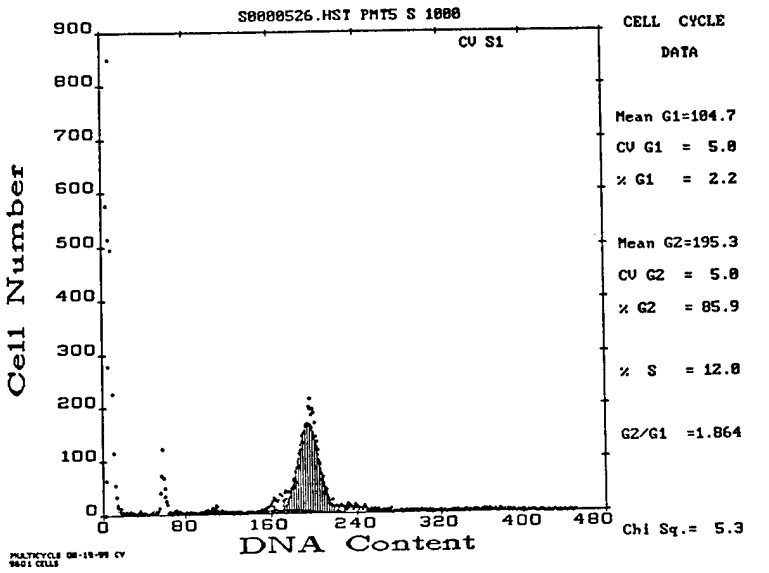






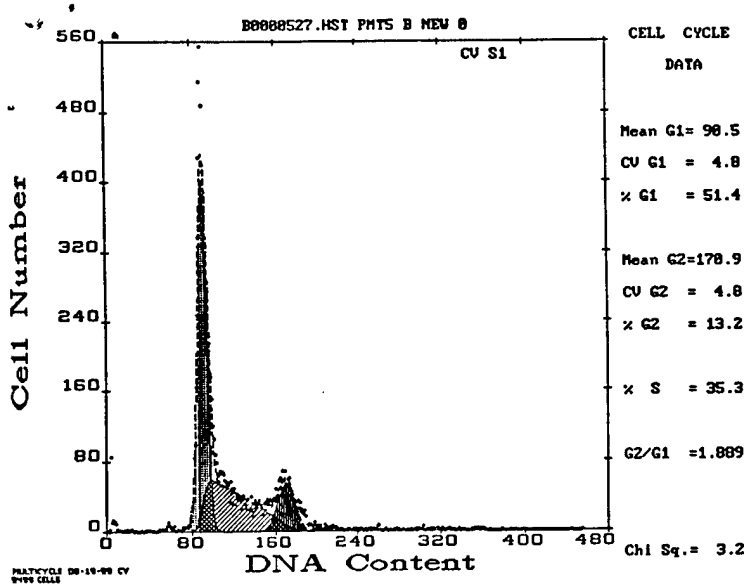






WILD TYPE

Appendix 2. Taxol Effect of
Wild-type and Mutant Transfectants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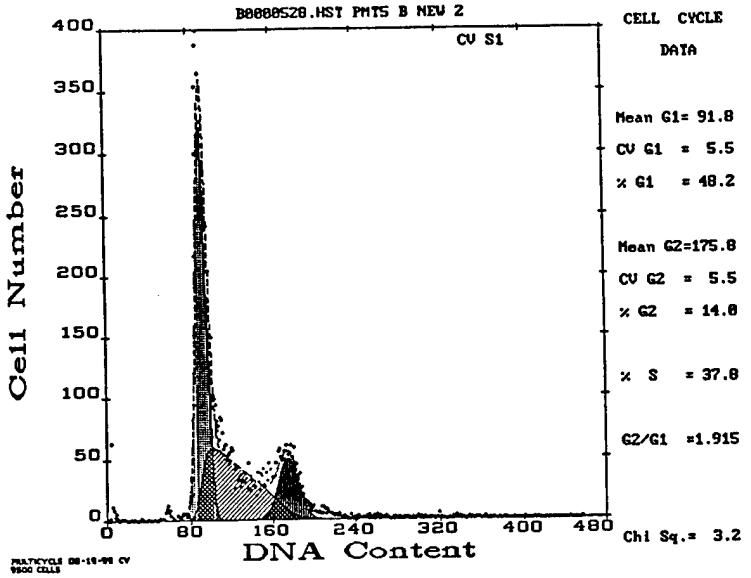
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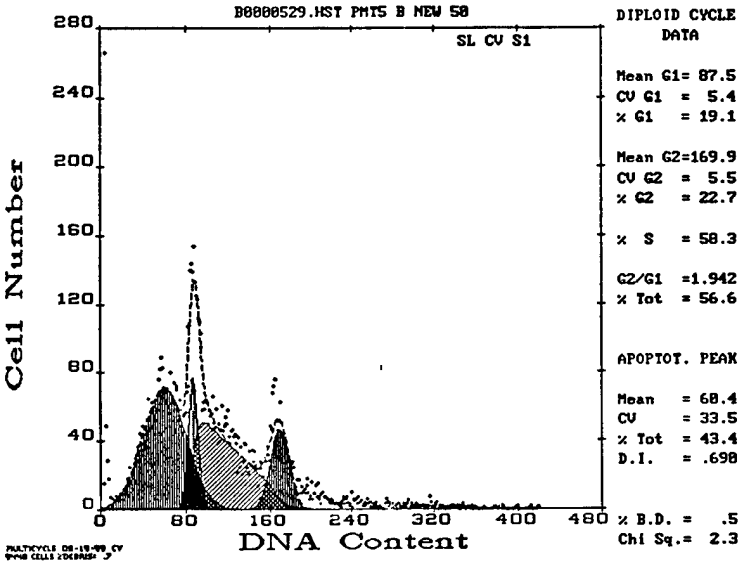
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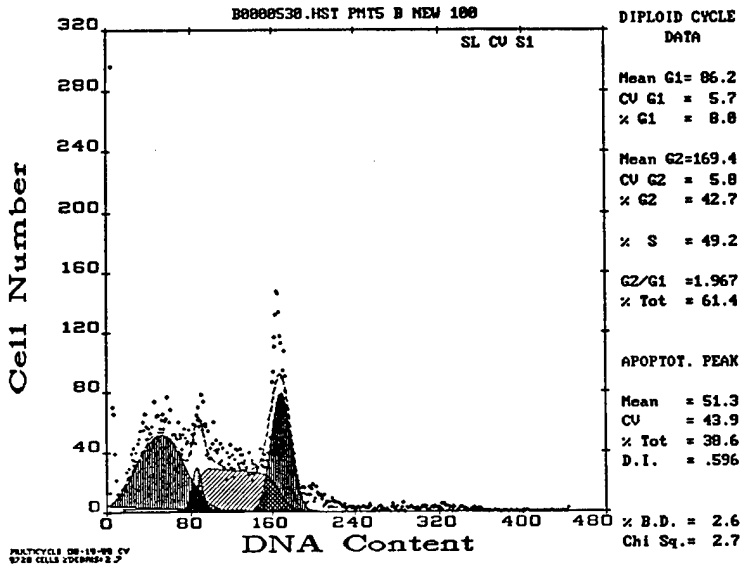
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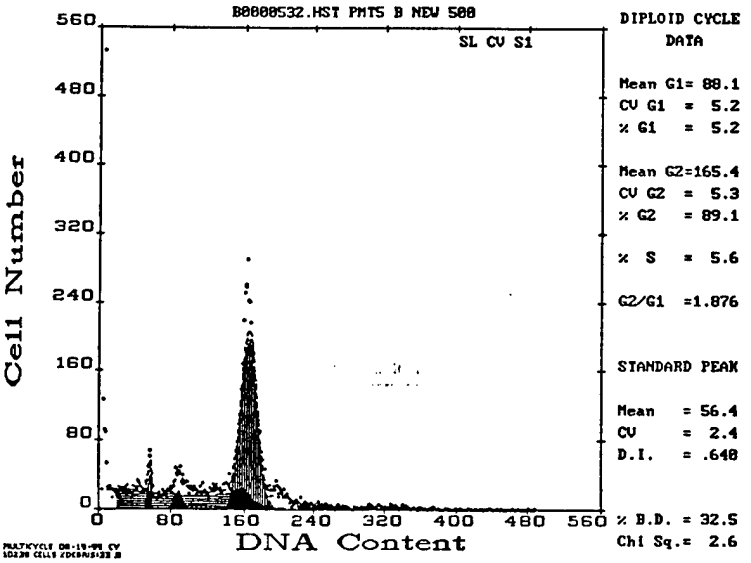
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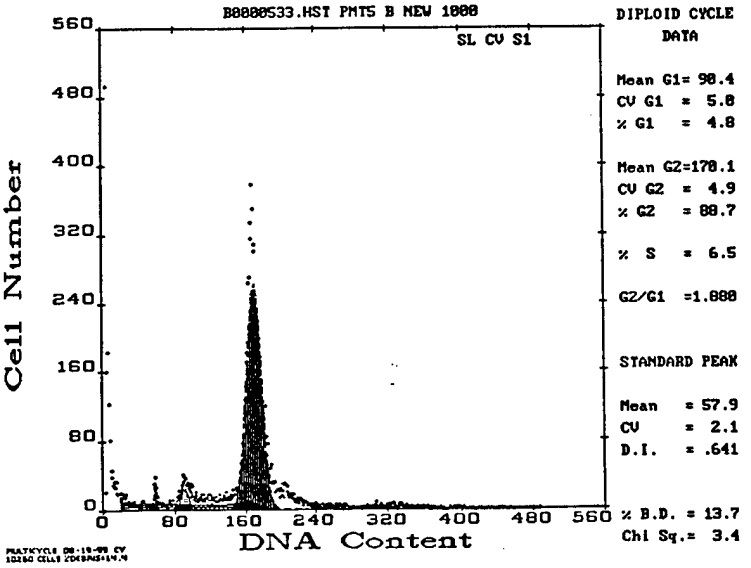
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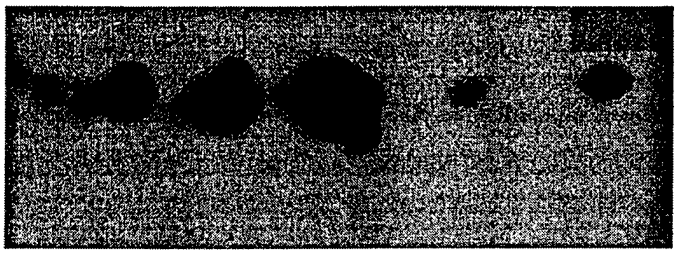
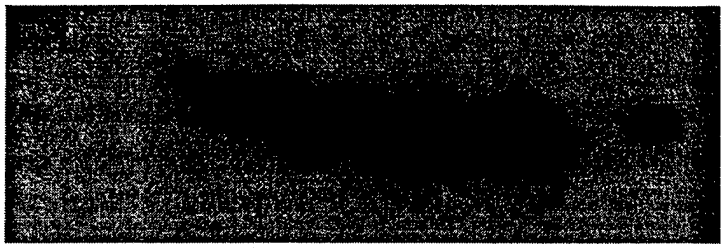
MUTANT

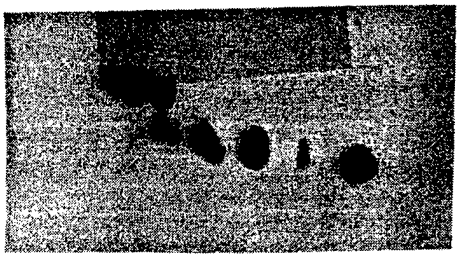
Sense

Mutant

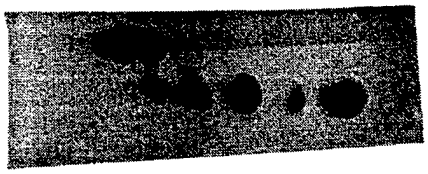


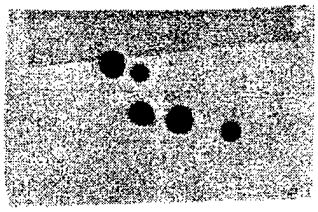


OP18

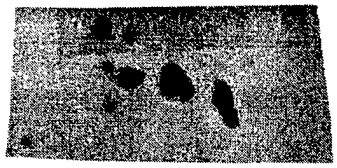


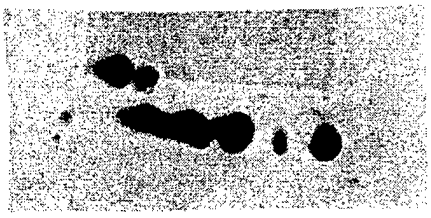
Serine 16



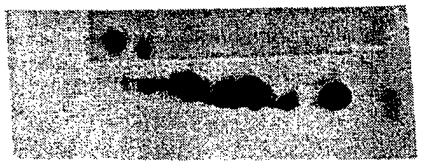


Serine 25





Serine 38



Appendix 3. Phosphorylation Status of Wild-type and Mutant Op18